

The genetic basis of C-glycosyl flavone B-ring modification in maize (*Zea mays* L.) silks

Moisés Cortés-Cruz, Maurice Snook, and Michael D. McMullen

Abstract: Resistance to corn earworm (CEW) (*Helicoverpa zea* Boddie) has been attributed to high concentrations of C-glycosyl flavones and chlorogenic acid in maize (*Zea mays* L.) silks. The most common C-glycosyl flavones isolated from maize silks are maysin, apimaysin, and methoxymaysin, which are distinguished by their B-ring substitutions. For a better understanding of the genetic mechanisms underlying the synthesis of these compounds, we conducted a quantitative trait locus (QTL) study with two populations: (Tx501 × NC7A)F₂ and (Tx501 × Mp708)F₂. For chlorogenic acid, maysin, and methoxymaysin concentration, the major QTL for both populations was located on chromosome 4 near *umc1963*. For apimaysin, the major QTL in both populations was located at the position of the *pr1* locus on chromosome 5. The QTL alleles on chromosome 4 that increased the synthesis of methoxymaysin significantly decreased the synthesis of maysin and chlorogenic acid. This decrease in maysin concentration was four-fold greater than the increase in methoxymaysin. Our results indicate that the QTL on chromosome 4, responsible for the increase in methoxymaysin synthesis, alters the dynamics of both the phenylpropanoid and flavonoid pathways.

Key words: *pr1*, flavonoid 3'-hydroxylase, maysin, apimaysin, methoxymaysin.

Résumé : La résistance au ver de l'épi du maïs (CEW) (*Helicoverpa zea* Boddie) a été attribuée à de fortes teneurs en flavones C-glycosylés et en acide chlorogénique dans les soies du maïs (*Zea mays* L.). Les flavones C-glycosylés les plus communément extraites des soies du maïs sont la maysine, l'apimaysine et la méthoxymaysine, lesquelles se distinguent les unes des autres par les substitutions au niveau de l'anneau B. Afin de mieux cerner les mécanismes génétiques qui sous-tendent la synthèse de ces composés, les auteurs ont réalisé une étude de locus à caractère quantitatif (QTL) sur deux populations : (Tx501 × NC7A)F₂ et (Tx501 × Mp708)F₂. En ce qui a trait aux teneurs en acide chlorogénique, en maysine et en méthoxymaysine, un QTL majeur a été identifié sur le chromosome 4 chez les deux populations, à proximité du marqueur *umc1963*. Pour l'apimaysine, le QTL principal chez les deux populations était situé au locus *pr1* sur le chromosome 5. Les allèles du locus QTL situé sur le chromosome 4 et qui accroissaient de manière significative la synthèse de méthoxymaysine diminuaient la synthèse de la maysine et de l'acide chlorogénique. Cette diminution de la synthèse de maysine était quatre fois plus considérable que l'accroissement observé pour la méthoxymaysine. Ces résultats indiquent que le locus QTL situé sur le chromosome 4, lequel est responsable d'un accroissement de la synthèse de méthoxymaysine, modifie la dynamique de la voie des phénylpropanoïdes et des flavonoïdes.

Mots clés : *pr1*, flavonoïde 3'-hydroxylase, maysine, apimaysine, méthoxymaysine.

[Traduit par la Rédaction]

Introduction

The corn earworm (CEW) (*Helicoverpa zea* Boddie) is a common generalist feeder with at least 16 cultivated (including maize (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), grain sorghum (*Sorghum bicolor* (L.) Moench), soybean (*Glycine*

max (L.) Merr.), tomato (*Lycopersicon esculentum* L.), alfalfa (*Medicago sativa* L.), peanuts (*Arachis hypogaea* L.), and other crops) and numerous (more than 100) wild hosts (Boyd and Bailey 2001). The larvae of CEW damage maize crops primarily by feeding in the tips of the ears, devouring kernels, and exposing the ear to possible microbial contamination. Fresh

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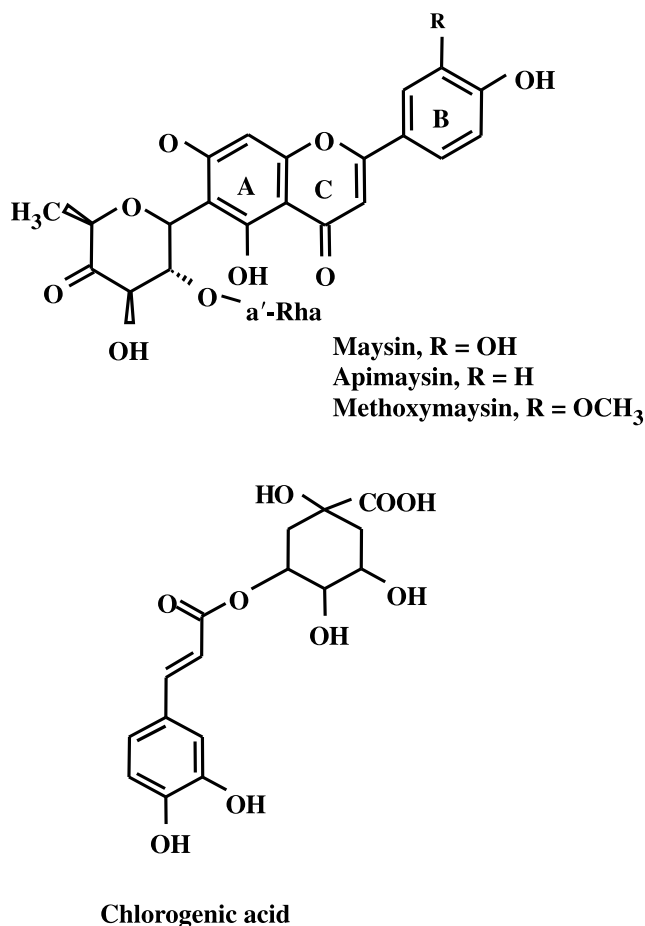
market and processing sweet corn and hybrid dent-seed corn are the major sectors of maize production affected by this insect. Although even severe infestations damage less than 10% of the kernels, this is enough to cause serious economic losses in sweet corn because of consumer rejection, and in hybrid dent-seed corn because of the high value of the crop (Delahaut and Wedberg 1996).

Recently, interest in the development of crops with natural resistance to insects has increased in response to environmental concerns and development of resistance to common pesticides (Snook et al. 1993). Natural resistance to CEW in maize has been attributed to the presence in the silks of the C-glycosyl flavone called maysin (2''-O- α -L-rhamnosyl-6-C-(6-deoxy-xylo-hexos-4-ulose)-luteolin) and related compounds: apimaysin (2''-O- α -L-rhamnosyl-6-C-(6-deoxy-xylo-hexos-4-ulose)-apigenin), methoxymaysin (2''-O- α -L-rhamnosyl-6-C-(6-deoxy-xylo-hexos-4-ulose)-3'-methoxyluteolin), and chlorogenic acid (a product of the phenylpropanoid pathway) (Fig. 1). Maysin is the most common compound isolated from maize silks, although apimaysin and methoxymaysin often occur in minor amounts (Widstrom et al. 1977; Waiss et al. 1979; Elliger et al. 1980a, 1980b; Snook et al. 1989, 1994; Wiseman et al. 1996; Widstrom and Snook 1997). The inbred line NC7A produces equal amounts of apimaysin and maysin, making it an ideal candidate to study the 3'-hydroxylation step in the synthesis of flavones of maize (Lee et al. 1998). Little is known about the genetic regulation of the synthesis of methoxymaysin. Similarly, the inbred line Tx501 accumulates high levels of methoxymaysin and this makes it a prime candidate for study as well.

The identification of maysin and maysin-related compounds as potent factors to CEW antibiosis has led to further studies to determine critical levels for activity. Waiss et al. (1981) demonstrated that a maysin level of 0.15% (w/w of diet) in laboratory bioassays reduced CEW larval mass by 50%. Wiseman et al. (1992) subsequently showed in a more detailed study that there was a highly significant negative relationship ($r = -0.81$, $P < 0.001$) between maysin concentration in fresh silks and mass of CEW larvae fed with diets containing methanol-silk extracts. In addition, their results showed that a silk maysin concentration of 0.2% (fresh weight) reduced larval mass by about 50% and that higher maysin levels ($>0.4\%$ fresh weight) reduced larval mass to about 70% of that of controls. Further studies carried out by Snook et al. (1994) compared the antibiosis activity of maysin, chlorogenic acid, apimaysin, and methoxymaysin. Their results showed that the activities of chlorogenic acid, apimaysin, and methoxymaysin were about half as effective in reducing CEW growth when compared with maysin. Elliger et al. (1980b) also reported that methoxymaysin had about half of the activity of maysin.

The C-glycosyl flavones function as antibiotic agents through their subsequent conversion to more toxic quinones, reducing the availability of free amino acids and proteins by binding to -SH and -NH₂ groups (Felton et al. 1989; Wiseman and Carpenter 1995). Toxicity of the common flavonoid glycosides requires vicinal hydroxyl groups on the B ring (present in maysin), a condition that is necessary, but not sufficient, for full biological activity (Elliger et al. 1980a, 1980b; Lindroth and Peterson 1988). Lower efficacy of the

Fig. 1. Chemical structures of the C-glycosyl flavones and chlorogenic acid.



monohydroxy flavones (apimaysin and methoxymaysin) is presumably the result of one less hydroxyl group available for oxidation during the conversion to quinones.

Byrne et al. (1996, 1997) showed that in a population segregating for functional and non-functional *p* alleles, the *p* region on the short arm of chromosome 1 behaves as a major quantitative trait locus (QTL) for maysin synthesis and CEW larval antibiosis. The *p* locus encodes a MYB-like transcription factor that regulates the synthesis of both phlobaphene pigmentation of maize pericarps and cob glumes and flavones in maize silks (Grotewold et al. 1994; McMullen et al. 1998). A second major QTL for maysin synthesis has been detected on the short arm of chromosome 9 (Byrne et al. 1996, 1998; Lee et al. 1998). This QTL (gene) has been designated *rem1* (*recessive enhancer of maysin 1*) and requires a functional *p* allele to increase maysin levels nearly two fold. In populations segregating for variation for both the *rem1* and *p* alleles, significant epistatic interactions between *p* and *rem1* alleles have been detected.

For apimaysin synthesis, a major QTL was detected in the long arm of chromosome 5 (explaining 65% of the phenotypic variance), consistent with the map position of the *pr1* (flavonoid 3'-hydroxylase) locus (Lee et al. 1998). The *pr1* locus is required for the 3' hydroxylation of anthocyanins in aleurone tissue (Larson et al. 1986). It has been assumed,

but never demonstrated, that the *prl* locus encodes the flavonoid 3'-hydroxylase enzyme. Structurally, maysin and apimaysin are highly related compounds, differing only by a 3'-hydroxyl group. Hence, based on the anthocyanin synthesis model in which all anthocyanins are synthesized from a common pathway, it was assumed that the synthesis of both compounds occurred via the same pathway (Fig. 2A), and that the *prl* locus would account for most of the variation in the levels of the two compounds (Styles and Ceska 1975). Surprisingly, the *prl* region controlling apimaysin levels did not have a significant effect on maysin synthesis. The *rem1* region, which affected maysin levels, did not alter apimaysin. These results suggested that the synthesis of these closely related compounds was at least partially independent (Lee et al. 1998; Fig. 2B). Lee et al. (1998) proposed metabolic channeling as a mechanism to explain the apparent independence of maysin and apimaysin synthesis.

Not only do these results apparently contradict the previous assumption, but they also pose additional questions about the role of *prl* in the synthesis of maysin and apimaysin. Are those results population specific or can we generalize them across populations? How can maysin be synthesized if *prl* is not involved? Are there other hydroxylase genes homologous to *prl* involved in the synthesis of these compounds? Larson et al. (1986) found that aleurone tissue homozygous for *prl* had no identifiable hydroxylase activity, but seedlings and sheaths homozygous for *prl* did have enzyme activity, suggesting the presence of other flavonoid hydroxylase genes in the maize genome. The inbred line Mp708 has the recessive, non-functional *prl* allele by test-cross to an aleurone anthocyanin tester yet makes almost all maysin and very little apimaysin (Lee et al. 1988). Our hypothesis is that there is an additional hydroxylase activity in the silks of the inbred Mp708 not found in NC7A, which compensates for the non-functional *prl* to synthesize maysin.

Furthermore, how does the synthesis of 3'-methoxymaysin relate to the synthesis of maysin and apimaysin? The simplest model would be presence of an *O*-methyltransferase that would add the methyl group to maysin, thus converting it to methoxymaysin (Fig. 2A). This hypothesis would predict that the genetic effects of a QTL that increases methoxymaysin would decrease maysin with a 1:1 effect.

In this paper, we report the results of a QTL study on the synthesis of the C-glycosyl flavone type in maize silks across two populations in which the parental lines segregate for functional (Tx501) vs. nonfunctional (NC7A and Mp708) *prl* alleles. Our objectives are to determine the chromosome regions associated with the synthesis of maysin, apimaysin, and methoxymaysin, and their genetic effects in the maize genome.

Materials and methods

Mapping populations

Population 1 was generated from a cross between inbred lines Tx501 and NC7A. Tx501 has moderately high methoxymaysin levels. NC7A has moderately high apimaysin, maysin, and chlorogenic acid levels in silk tissues. Tx501 has a functional *Pr1* allele (based on phenotypic observations of test crosses for aleurone color), and a *P-wwb* (color-

less pericarp, white cob, browning silks) constitution at the *p* locus. NC7A has a non-functional *prl* allele, a *P-wwb* allele at the *p* locus, and a *rem1* allele that increases maysin (Byrne et al. 1996). Population 2 was generated from a cross between the inbreds Tx501 and Mp708. Mp708 has moderately high maysin and chlorogenic acid levels in silk tissues, a non-functional *prl* allele, and a *P-wwb* constitution at the *p* locus. For both populations, F₂ individuals were derived from two self-pollinated F₁ plants.

Tissue collection and chemical analysis

Three hundred forty-four (Tx501 × Mp708)F₂ and 264 (Tx501 × Mp708)F₂ plants were grown at the University of Missouri Bradford Research and Extension Center near Columbia, Mo., during the summers of 1998 and 1999, respectively. In both years, two replications of parental and F₁ seeds were grown in rows adjacent to the F₂. Leaf tissue was collected from parental, F₁, and individual F₂ plants at the midwhorl stage for simple sequence repeat (SSR) analysis. Ear shoots were covered before silk emergence to prevent pollination. Silk tissue was collected two days after silk emergence. Lyophilized samples were shipped to the Richard B. Russell Research Center, Athens, Ga., for chemical analysis. The lyophilized silk masses were extracted with 120 mL methanol at 0°C for 14 days. Extract concentrations of chlorogenic acid, maysin, apimaysin, and methoxymaysin were determined by reversed-phase high performance liquid chromatography (Snook et al. 1989, 1993) and expressed in percent fresh silk weight.

SSR analysis

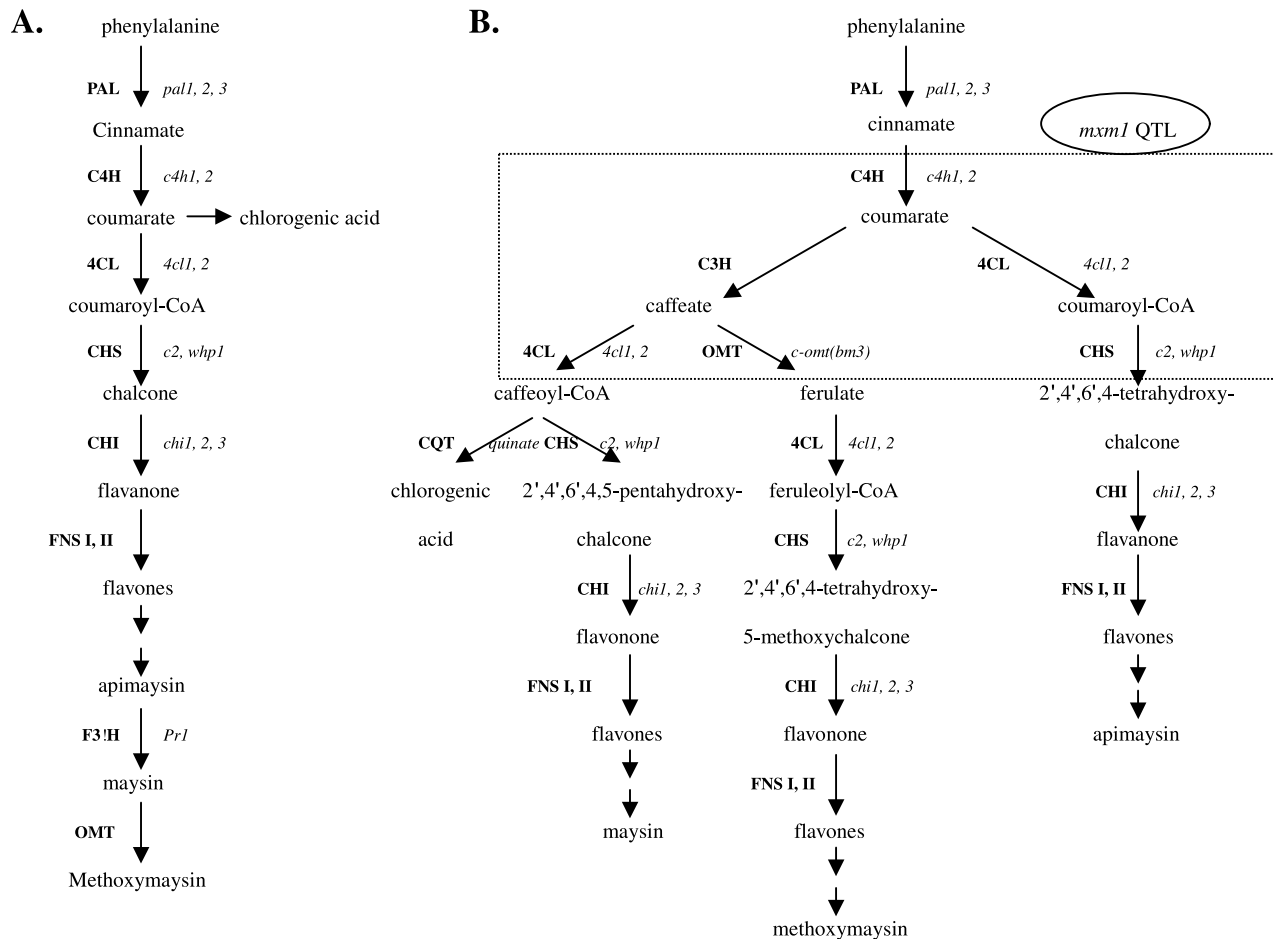
Genomic DNA was extracted from lyophilized, ground leaf tissue by the mixed cetyltrimethylammonium bromide (CTAB) procedure (Saghai-Marooof et al. 1984). One hundred twenty and 116 SSR primers were used in populations 1 and 2, respectively. Polymerase chain reaction (PCR) conditions for SSR markers were as follows: 50 ng of each primer, 0.3 U Platinum *Taq* polymerase (Invitrogen, Carlsbad, Calif.), 1× Platinum *Taq* buffer, 50 ng genomic DNA, 2.5 mM MgCl₂, 0.4 mM of each dNTP, and sterile H₂O added to obtain a total volume of 15 µL. Cycling conditions were as follows: 10 cycles of 1 min at 95°C, 1 min at 65°C, and 1.5 min at 72°C, with a 1°C decrement of annealing temperature in each cycle until it reached 55°C, followed by 25 cycles of 1 min at 95°C, 1 min at 55°C, and 1.5 min at 72°C. Reactions were carried out in 96-well plates (Fisher Scientific, St. Louis, Mo.) with a tetrad thermal cycler (PTC-225, MJ Research, Watertown, Mass.).

The PCR products were electrophoresed on 3% w/v SFR™ agarose gels (Amresco, Solon, Ohio), with a constant voltage of 115 V until the polymorphic products were clearly separated. The SSR markers were selected to provide coverage of the entire maize genome when possible, at approximately 20-cM intervals (MaizeDB, available from <http://www.agron.missouri.edu/ssr.html>).

Statistical analysis

Segregation at each marker locus was checked for significant ($P < 0.001$) deviations from the expected Mendelian segregation ratio (1:2:1) using a standard χ^2 test. Genetic linkage maps were constructed using MAPMAKER/EXP

Fig. 2. Phenylpropanoid–flavonoid pathway in maize for the synthesis of chlorogenic acid and the C-glycosyl flavones. (A) Simplest proposed pathway. (B) Alternative pathway with specific chalcone precursors for the synthesis of maysin, apimaysin, and methoxymaysin. The chemical class is indicated in lower case letters. Enzyme abbreviations are as follows: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; C3H, coumarate 3-hydroxylase; 4CL, 4-coumarate–CoA ligase; CQT, coumaroyl CoA : quinate hydroxycinnamoyl transferase; CHS, chalcone synthase; CHI, chalcone isomerase; FNS, flavone synthase; OMT, *O*-methyltransferase; and F3'H, flavonoid 3'-hydroxylase. Loci are shown in *italics*. The dashed box indicates the potential points of activity of the *mxm1* QTL.

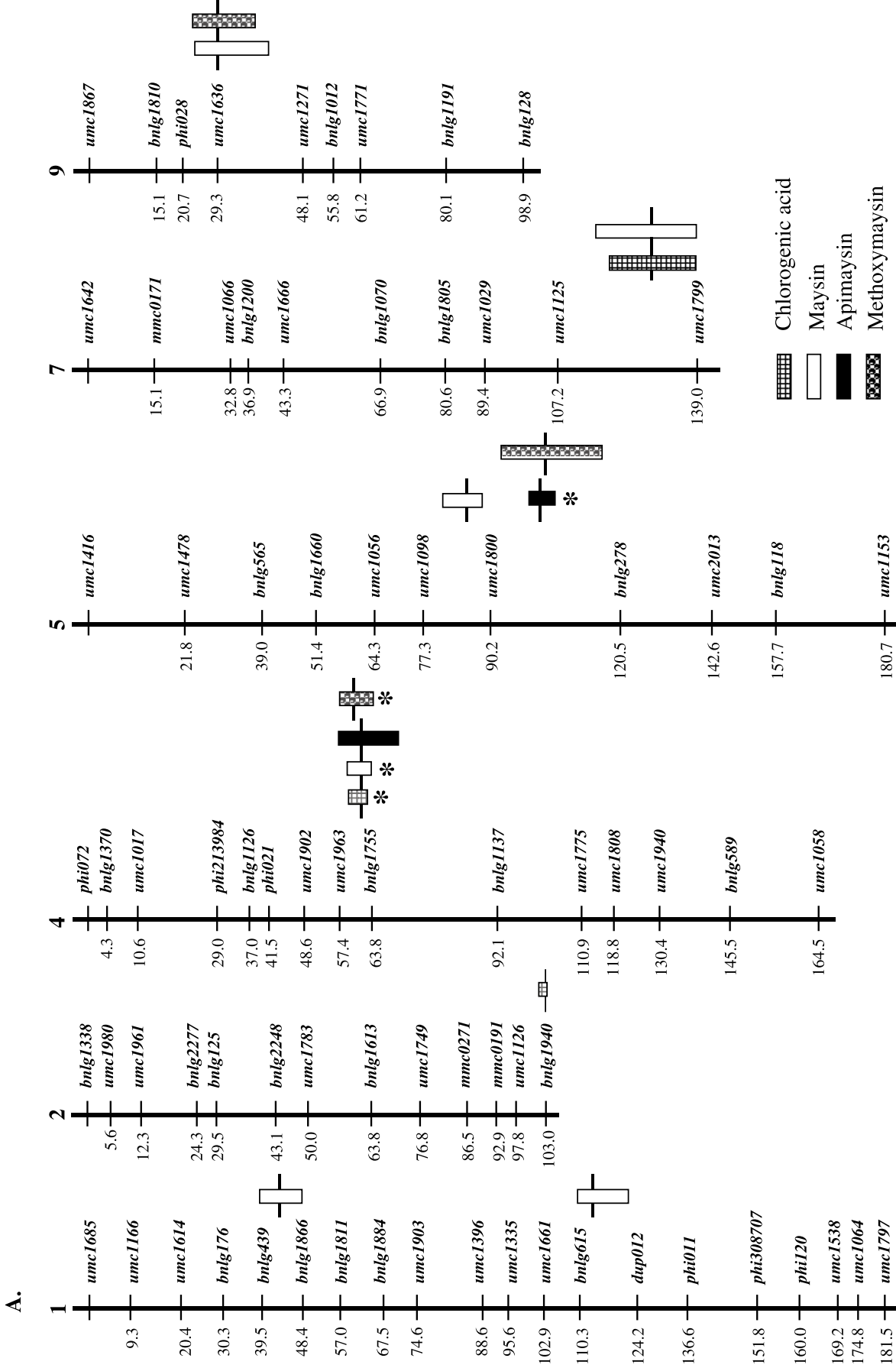


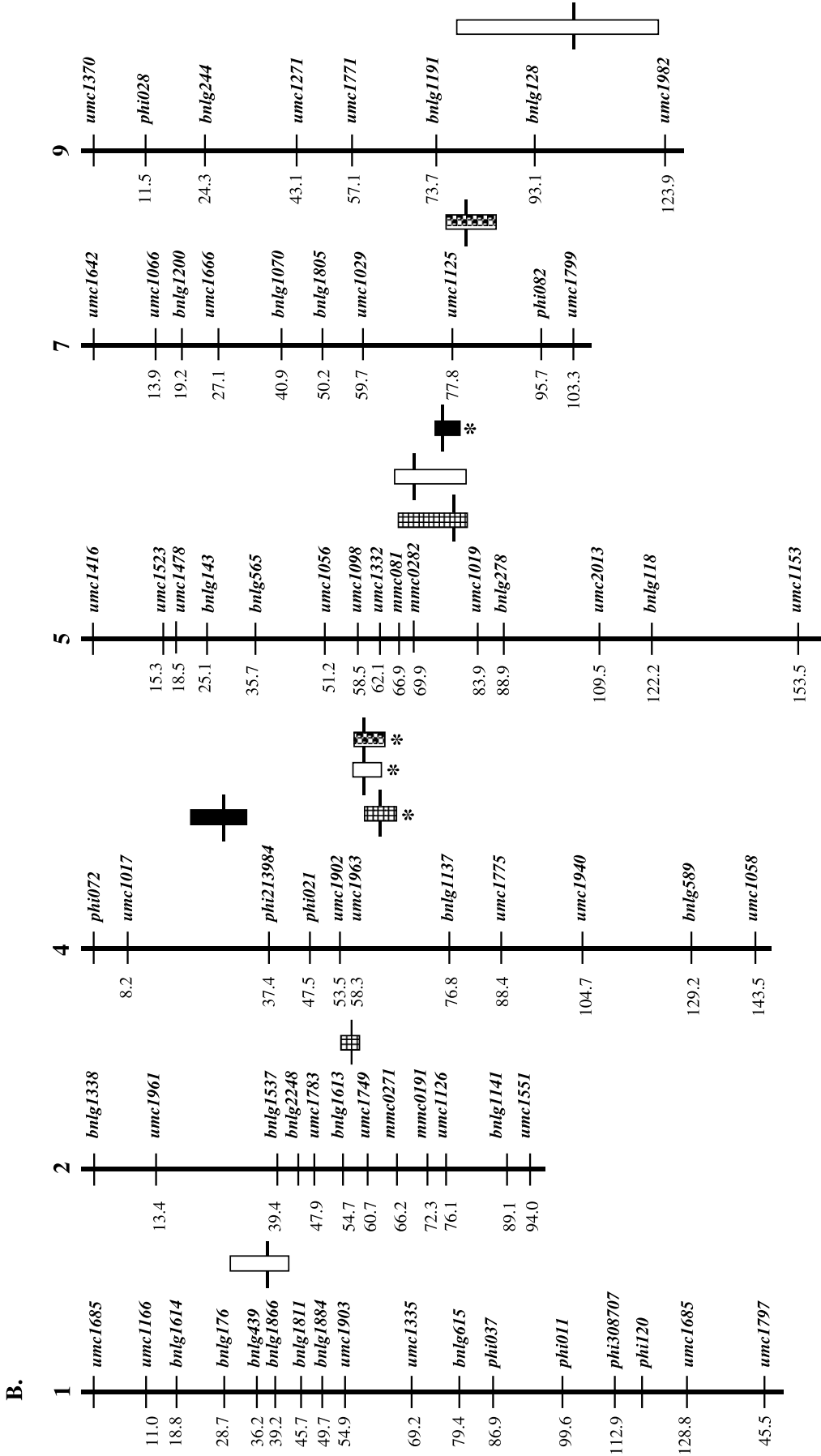
version 3.0b (Whitehead Institute, Cambridge, Mass.) for UNIX, with a minimum likelihood of odds (LOD) score of 3.0 and a maximum distance of 60 cM. Deviation from normality for phenotypic trait data for both F₂ populations was tested using the Shapiro–Wilk statistic with PROC UNIVARIATE of SAS (SAS Institute Inc. 1990). Detection of the QTLs affecting chlorogenic acid, maysin, apimaysin, and methoxymaysin levels was performed by composite interval mapping (CIM) using QTL Cartographer version 1.15 (Basten et al. 1994, 2001) on UNIX and PC platforms. The identification of cofactors for the CIM analysis was done using the SRMAPQTL function, using forward–backward regression with $p(F\text{-in})$ and $p(F\text{-out}) = 0.01$. The ZMAPQTL function was used for single-trait mapping, using a total of 6, 10, 4, and 7 cofactors in population 1 for the traits chlorogenic acid, maysin, apimaysin, and methoxymaysin, respectively. For population 2, the number of cofactors used was 8, 8, 3, and 5 for the same traits. The genetic effects were calculated using the ZMAPQTL function under the hypothesis of $H_3: H_0; a \neq 0, d \neq 0$. Critical threshold levels for declaring a QTL significant were determined empirically by permuta-

tion analysis (1000 permutations) to give a genome-wide error rate of 0.05 (Churchill and Doerge 1994; Doerge and Churchill 1996). The critical values for chlorogenic acid, maysin, apimaysin, and methoxymaysin were as follows: for population 1, likelihood ratios (LR) of 16.08 (LOD = 3.50), 16.94 (LOD = 3.68), 16.43 (LOD = 3.57), and 16.28 (LOD = 3.52); for population 2, LR of 16.44 (LOD = 3.57), 15.54 (LOD = 3.37), 16.25 (LOD = 3.53), and 16.25 (LOD = 3.53), respectively.

Significant ($P < 0.001$) two-way epistatic interactions were identified using EPISTACY (Holland 1998). Stepwise selection was performed with PROC REG (SAS 1990) to construct a multiple-locus model (MLM). Significant ($P < 0.001$) single loci identified by the SRMAPQTL function and two-way interactions from EPISTACY were included in the model (excluding closely linked loci). The best model was determined to be that which explained the greatest proportion of the phenotypic variance and in which single loci and two-way interactions were significant at the 0.05 significance level. Furthermore, single loci from the epistatic interactions were included in the model and retained if the two-

Fig. 3. Genetic linkage maps for (A) (Tx501 × NC7A)F₂ and (B) (Tx501 × Mp708)F₂ populations. Only linkage groups with significant QTLs are shown. The locus names are as given in MaizeDB (www.agron.missouri.edu). Distances are given in centimorgans and are cumulative. The positions of the QTLs are shown by bars. A line through the bars indicates the peak likelihood position and the extent of the bar represents a one-LOD confidence interval for the QTL position. Bars with an asterisk indicate the main QTL for the trait.





way interaction remained significant ($P > 0.05$); otherwise, they were eliminated and the R^2 value from the model was recalculated. The LSMEANS option from SAS (SAS Institute Inc. 1990) was used to calculate genotypic class means for chlorogenic acid, maysin, apimaysin, and methoxymaysin.

The genotypic and phenotypic information used in the analysis of this paper will be deposited in MaizeDB (www.agron.missouri.edu) for public access.

Results

Population 1 ((Tx501 × Mp708)F₂)

Distribution of the C-glycosyl flavone concentrations of the F₂ plants was not normal because of a large number of low values and a long tail for high values, especially for maysin and apimaysin (data not shown). Transgressive segregation was observed for these compounds, as well as for chlorogenic acid. All F₂ individuals contained appreciable amounts of maysin, whereas only one quarter of the individuals contained >0.015% apimaysin, suggesting that this compound may be under recessive, single-gene control as reported previously by Lee et al. (1998).

The framework linkage map from 346 F₂ individuals included 120 loci with a total genome length of 1350 cM. The average interval length between loci was 11.2 cM. Segregation ratios were severely distorted ($P < 0.001$) for genotypes of several loci located on the short arm of chromosome 4 (*bnlg1370*, *phi072*, *umc1017*, *phi021*, *umc1902*, and *umc1963*) and chromosome 3 (*bnlg1647*). This distortion was in favor of alleles from NC7A.

Composite interval mapping by QTL Cartographer was used to identify significant QTLs for chlorogenic acid, maysin, apimaysin, and methoxymaysin (Fig. 3A and Table 1). The major QTL for the synthesis of chlorogenic acid, maysin, and methoxymaysin was detected on chromosome 4, near *umc1963*. At this particular locus, dominant to partially dominant gene action was present, with the homozygous alleles of Tx501 causing an increase in methoxymaysin concentration and a decrease in chlorogenic acid and maysin concentrations (Table 2). We have designated this locus *methoxymaysin1* (*mxm1*). A major QTL affecting the synthesis of apimaysin was detected on chromosome 5, consistent with the map position of the *pr1* locus. The QTL in this region accounted for 79.0% of the phenotypic variation for apimaysin. Dominant gene action for low apimaysin was observed and was consistent with the expectation that a recessive, nonfunctional *pr1* allele is required for apimaysin accumulation (Lee et al. 1998).

Significant ($P < 0.001$) gene-interaction effects were detected using the program EPISTACY (Holland 1998) for maysin, apimaysin, and methoxymaysin. The MLM for maysin included six individual loci and one epistatic interaction (*bnlg439* × *umc1636*) and accounted for 44.8% of the phenotypic variance (Table 3). The *bnlg439* locus mapped within the *p* region on chromosome 1 and the locus *umc1636* mapped at the *rem1* region. All the single-locus terms were identified as QTL with major and minor effects for the synthesis of maysin, except for the region on the long arm of chromosome 5 (*bnlg118*, bin 5.07). The 10 maize chromosomes are subdivided into bins, which are regions approximately 20 cM in length. The chromosome number is

noted on the left-hand side of the decimal and the bin is denoted on the right-hand side (MaizeDB).

The MLM for apimaysin in this population included two individual loci and their epistatic interaction and accounted for 33.3% of the phenotypic variance. The epistatic interaction involved the two loci detected as QTL with main effects, *umc1800* and *umc1963*. The *umc1880* locus mapped within the *pr1* region on the short arm of chromosome 5, whereas the main QTL for chlorogenic acid, maysin, and methoxymaysin (*mxm1*) mapped near the *umc1963* locus. The MLM for methoxymaysin included three individual loci and one epistatic interaction between the *umc1963* (*mxm1*) and *umc1636* (*rem1*) loci regions. This model accounted for 33.0% of the phenotypic variance. Both terms were detected as significant loci for methoxymaysin QTLs.

Population 2 ((Tx501 × Mp708)F₂)

Distribution of the C-glycosyl flavone concentrations of the F₂ plants was not normal because of a large number of low values and a long tail of high values, especially for maysin and methoxymaysin. Transgressive segregation was observed for chlorogenic acid, maysin, and apimaysin.

A linkage map based on 246 F₂ individuals and 116 SSR markers covered a total genome length of 1179 cM. The average interval length between loci was 10.2 cM. Chi-square analysis detected severe segregation distortion ($P < 0.001$) for genotypes of loci located on the short arm of chromosome 4 (*phi072*, *umc1017*, *phi213984*, *phi021*, *umc1902*, and *umc1963*) in favor of alleles from Mp708.

As in population 1, the QTL with the largest effects for the synthesis of chlorogenic acid, maysin, and methoxymaysin was detected on chromosome 4, near the *umc1963* locus. In this region, partially dominant gene action was present, with the homozygous alleles of Tx501 again causing an increase in methoxymaysin concentration and a decrease in chlorogenic acid and maysin concentrations (Table 2). For the synthesis of apimaysin, the major QTL detected was on chromosome 5, near *mmc0282*, at the *pr1* region (Fig. 3B). This QTL accounted for 80.8% of the phenotypic variation of apimaysin. Dominant gene action was detected in this region for low apimaysin accumulation. Unlike Lee et al. (1988), the QTL for apimaysin on chromosome 5 was also significant for maysin synthesis in populations 1 and 2. Furthermore, the QTL for maysin synthesis on chromosome 4 was significant for apimaysin levels in population 1 (Table 1).

Significant ($P < 0.001$) epistatic interactions for maysin, apimaysin, and methoxymaysin were detected by EPISTACY (Holland 1998). The MLM for maysin included five individual loci and one epistatic interaction, *umc1963* (*mxm1*) × *umc1125* (bin7.04). This model accounted for 37.1% of the phenotypic variance (Table 4). All the loci in the model were detected as significant QTLs for maysin synthesis, except for the *umc1125* locus. This locus was, however, detected as a significant QTL for maysin synthesis in population 1.

The MLM for apimaysin included two individual loci and an epistatic interaction, *mmc0282* × *phi126*. This model accounted for 44.1% of the phenotypic variance. The *mmc0282* locus mapped within the *pr1* region on the short arm of chromosome 5. The second term of the interaction included a locus that was not identified as a QTL, *phi126*, on the

Table 1. Quantitative trait loci (QTLs) detected by composite interval mapping for chlorogenic acid, maysin, apimaysin, and methoxymaysin concentrations in maize silks in the (Tx501 × NC7A)_F₂ and (Tx501 × Mp708)_F₂ populations.

Trait	Bin	Marker	QTL position*	$(R^2)^{\dagger}$	Likelihood ratio	Genetic effects [‡]		Gene action [§]
						A	D	
(Tx501 × NC7A)F ₂								
Chlorogenic acid	2.08	<i>bnlg1940</i>	103.0	2.7	18.1	0.010	0.004	PD
	4.04	<i>umc1963</i>	61.3	51.1	276.2	−0.044	−0.030	PD
	7.04	<i>umc1125</i>	127.2	5.7	23.2	−0.013	0.013	D
Maysin	1.03	<i>bnlg439</i>	43.5	8.5	50.4	0.084	0.036	PD
	1.07	<i>bnlg615</i>	114.5	5.3	30.9	0.064	0.011	A
	4.04	<i>umc1963</i>	61.3	35.2	209.0	−0.148	−0.120	D
	5.04	<i>umc1098</i>	85.3	4.3	25.2	0.058	−0.016	PD
	7.04	<i>umc1125</i>	127.2	4.8	20.5	−0.060	0.022	PD
	9.02	<i>umc1636</i>	29.3	3.0	20.7	−0.047	−0.006	A
Apimaysin	4.04	<i>umc1963</i>	61.3	3.5	17.0	−0.019	−0.023	D
	5.05	<i>umc1800</i>	102.2	78.9	392.3	−0.122	0.115	D
Methoxymaysin	4.04	<i>umc1963</i>	59.3	31.5	139.9	0.028	0.018	PD
	5.05	<i>umc1800</i>	104.2	8.1	22.4	0.010	−0.017	OD
	9.02	<i>umc1636</i>	29.3	4.1	21.2	−0.010	−0.001	A
(Tx501 × Mp708)F ₂								
Chlorogenic acid	2.04	<i>bnlg1613</i>	56.7	25.7	125.0	−0.037	0.015	PD
	4.04	<i>umc1963</i>	62.2	35.7	186.9	−0.056	0.038	PD
	5.05	<i>mmc0282</i>	77.9	4.3	21.8	0.015	0.005	PD
Maysin	1.03	<i>bnlg439</i>	38.1	7.7	32.9	0.043	−0.027	PD
	4.04	<i>umc1963</i>	60.2	32.7	119.0	−0.124	0.083	PD
	5.05	<i>mmc0282</i>	69.9	5.3	23.3	0.037	0.023	PD
	9.07	<i>bnlg128</i>	103.1	5.0	16.5	0.039	0.021	PD
Apimaysin	4.01	<i>umc1017</i>	28.1	30.7	51.9	0.066	−0.078	D
	5.05	<i>mmc0282</i>	75.9	80.8	308.3	−0.065	−0.061	D
Methoxymaysin	4.04	<i>umc1963</i>	60.2	67.8	313.7	0.037	−0.030	PD
	7.04	<i>umc1125</i>	79.9	4.5	19.5	−0.009	−0.002	A

*QTL position is cumulative starting with the most distal marker on the short arm.

[†]Percent of the phenotypic variance explained by each QTL. Values are expressed as percentages of fresh silk weight.

[‡]Genetic effects are calculated under the hypothesis of $H_3:H_0$; $a \neq 0$, $d \neq 0$. The direction of the genetic effects is give relative to the Tx501 allele.

[§]Calculated as |dominance effect/additive effect|: additive (A) = 0.0–0.2; partially dominant (PD) = 0.2–0.8; dominant (D) = 0.8–1.2; overdominant (OD) ≥ 1.2.

short arm of chromosome 6 (bin 6.00). There are no candidate loci involved in the flavonoid pathway at this region; however, QTLs for maysin concentration (*qmaysin9*) and response to CEW (*qcew9*) have been identified within this bin (Byrne et al. 1998). The MLM for methoxymaysin included three individual loci and two epistatic interactions and accounted for 51.4% of the phenotypic variance. All of the loci in this model were identified as QTLs except for *umc1370*, which is located on the short arm of chromosome 9 (bin 9.01, near the *rem1* region).

Discussion

QTLs for C-glycosyl flavones and chlorogenic acid

Populations 1 and 2 segregate for functional and nonfunc-

tional *prl* alleles. As expected, the *prl* region on chromosome 5 had the largest effect on apimaysin concentration by far, confirming its importance as a QTL for apimaysin synthesis as previously reported (Lee et al. 1998). This region accounted for 79 and 81% of the phenotypic variance for apimaysin concentration in populations 1 and 2, respectively. In population 1, the effect of the NC7A alleles was dominant for low apimaysin, with apimaysin mean concentrations of 0.006, 0.013, and 0.150% of fresh silk weight for the Tx501 homozygous, Tx501/NC7A heterozygous, and NC7A homozygous classes, respectively. For population 2, the same type of gene action was detected for the homozygous alleles from Mp708. The mean apimaysin concentrations for Tx501 homozygous, Tx501/Mp708 heterozygous, and Mp708 homozygous were 0.008, 0.010, and 0.099% of

Table 2. Genotype class means for chlorogenic acid, maysin, apimaysin, and methoxymaysin concentrations at the major QTL positions for the (Tx501 × NC7A)F₂ and (Tx501 × Mp708)F₂ populations.

Population	Genotype class	QTL	Fresh silk weight (%)			
			Chlorogenic Ac.	Maysin	Apimaysin	Methoxy-maysin
(Tx501 × NC7A)F ₂						
	Tx501	<i>umc1963 (mxm1)</i>	0.040a	0.082a		0.083a
	H	<i>umc1963 (mxm1)</i>	0.105b	0.347b		0.043b
	NC7A	<i>umc1963 (mxm1)</i>	0.124c	0.364b		0.032c
	Tx501	<i>umc1800 (pr1)</i>		0.329a	0.005a	
	H	<i>umc1800 (pr1)</i>		0.306a	0.012b	
	NC7A	<i>umc1800 (pr1)</i>		0.249b	0.149c	
(Tx501 × Mp708)F ₂						
	Tx501	<i>umc1963 (mxm1)</i>	0.035a	0.077a		0.091a
	H	<i>umc1963 (mxm1)</i>	0.123b	0.264b		0.031b
	Mp708	<i>umc1963 (mxm1)</i>	0.152c	0.314c		0.021c
	Tx501	<i>mmc0282 (pr1)</i>		0.299a	0.008a	
	H	<i>mmc0282 (pr1)</i>		0.267b	0.010b	
	Mp708	<i>mmc0282 (pr1)</i>		0.222c	0.098 ^c	

Note: Within each QTL, means followed by the same letter are not significantly different at $\alpha = 0.05$. H, heterozygote.

Table 3. Multiple locus models for chlorogenic acid, maysin, apimaysin, and methoxymaysin concentrations in the (Tx501 × NC7A)F₂ population.

Trait	(R ²)*	Locus or interaction†	Bin	Significance (P<)
Chlorogenic acid	43.2	<i>mxm1</i>	4.04	0.0001
		<i>bnlg1940</i>	2.08	0.0002
		<i>umc1125</i>	7.04	0.0144
Maysin	44.8	<i>mxm1</i>	4.04	0.0001
		<i>bnlg615</i>	1.07	0.0001
		<i>rem1</i>	9.02	0.0001
		<i>bnlg118</i>	5.07	0.0070
		<i>umc1125</i>	7.04	0.0287
		<i>p</i>	1.03	n.s.
		<i>p × rem1</i>	1.03 × 9.02	0.0011
Apimaysin	33.3	<i>pr1</i>	5.05	0.0001
		<i>mxm1</i>	4.04	n.s.
		<i>pr1 × mxm1</i>	5.05 × 4.04	0.0020
Methoxymaysin	36.3	<i>pr1</i>	5.05	0.0001
		<i>mxm1</i>	4.04	0.0001
		<i>rem1</i>	9.02	n.s.
		<i>mxm1 × rem1</i>	4.04 × 9.02	0.0008

*The percentage of the phenotypic variation explained by the model.

†Marker loci included in the models were selected using stepwise selection performed with PROC REG (SAS Institute Inc. 1990), using $P < 0.05$ as the significance threshold. Interactions were initially identified with EPISTACY ($P < 0.001$). Loci involved in significant interactions were included regardless of significance of main effect.

fresh silk weight. Unlike other studies, a minor QTL affecting the synthesis of maysin was also detected at the *pr1* region. This outcome shows that the synthesis of both compounds does not occur totally independently as previously reported, although their synthesis ratio is not reciprocal. Apimaysin was synthesized only in individuals homozygous for a nonfunctional *pr1* allele, which significantly decreased maysin levels in these individuals. However, the increase in apimaysin levels was two-fold greater than the decrease in maysin. The additive gene effects for *pr1* were 0.058% of fresh weight for maysin vs. -0.122% of fresh weight for apimaysin in population 1 and 0.037% of fresh weight for maysin vs. -0.065% of fresh weight for apimaysin in population 2 (Table 1). Additionally, high levels

of maysin were detected in all genotype classes, suggesting the presence of alternative hydroxylase genes involved in the synthesis of maysin (Table 2). We hoped to detect a second locus for apimaysin that would correspond to a duplicate hydroxylase gene. We were unsuccessful, because the only other main-effect QTL for apimaysin in either population was the major QTL for methoxymaysin on chromosome 4 (*mxm1*). The genetic effects of this locus are inconsistent with that of a second hydroxylase gene because the Tx501 allele of *mxm1* decreased the levels of apimaysin. This is the opposite of the expected effect if it were uncovering the compensating hydroxylase from Mp708. Our inability to identify a second apimaysin QTL consistent with a hydroxylase might be because of limited differences between QTL

Table 4. Multiple locus models for chlorogenic acid, maysin, apimaysin, and methoxymaysin concentrations in the (Tx501 × Mp708)F₂ population.

Trait	(R ²)*	Locus or interaction†	Bin	Significance (P<)
Chlorogenic acid	35.6	<i>mxm1</i>	4.04	0.0001
		<i>bnlg1613</i>	2.04	0.0001
		<i>pr1</i>	5.05	0.0005
Maysin	37.1	<i>p</i>	1.03	0.0001
		<i>bnlg128</i>	9.07	0.0001
		<i>pr1</i>	5.05	0.0001
		<i>umc1125</i>	7.04	0.0135
		<i>mxm1</i>	4.04	0.0136
		<i>mxm1</i> × <i>umc1125</i>	4.04 × 7.04	0.0140
		<i>pr1</i>	5.05	0.0001
Apimaysin	44.1	<i>qmaysin9</i>	6.00	n.s.
		<i>pr1</i> × <i>qmaysin9</i>	5.05 × 6.00	0.0001
		<i>mxm1</i>	4.04	0.0001
Methoxymaysin	51.4	<i>umc1125</i>	7.04	0.0001
		<i>rem1</i>	9.01	0.0001
		<i>mxm1</i> × <i>rem1</i>	4.04 × 9.01	0.0001
		<i>mxm1</i> × <i>umc1125</i>	4.04 × 7.04	0.0001

*The percentage of the phenotypic variation explained by the model.

†Marker loci included in the models were selected using stepwise selection performed with PROC REG (SAS Institute Inc. 1990), using $P < 0.05$ as the significance threshold. Interactions were initially identified with EPISTACY ($P < 0.001$). Loci involved in significant interactions were included regardless of significance of main effect.

alleles of the parents at this locus and because any effect of this QTL on apimaysin would only be expressed in one quarter of individuals of the population homozygous recessive for *pr1*.

The novel QTL on chromosome 4 showed pleiotropic effects for the synthesis of chlorogenic acid, maysin, and methoxymaysin. Homozygous alleles from Tx501 significantly increased the synthesis of methoxymaysin in both populations, and this increase in methoxymaysin levels was characterized by a highly significant decrease in chlorogenic acid and maysin (Table 2). In fact, the decrease in both compounds, chlorogenic acid and maysin, was much greater than the increase in the methoxymaysin level. The decrease in maysin vs. increase in methoxymaysin was 5.3 fold for population 1 and 3.4 fold for population 2 (see additive gene effects, Table 1). According to our data, this QTL is positioned near the *umc1963* locus on the short arm of chromosome 4 and, unlike the QTL for apimaysin synthesis on chromosome 5, it is not closely linked to any reported flavonoid pathway locus. In both populations, there was essentially recessive gene action for the Tx501 allele for high methoxymaysin levels and for low levels of both chlorogenic acid and maysin (Table 2).

The difference in chemical structure between maysin and methoxymaysin is solely an additional methyl group added to the 3'-hydroxyl of the B-ring of maysin. Anticipating the simplest genetic model, we expected these QTL studies to identify loci encoding or regulating the expression of a methyltransferase that would add the methyl group to maysin (Fig. 2A). The pleiotropic effects of the *mxm1* locus are inconsistent with this genetic model. That *mxm1* reduces maysin by an average of four fold compared with the methoxymaysin produced and also causes a large decrease in chlorogenic acid, which indicates that *mxm1* is not involved in a single enzymatic reaction late in the pathway, but rather

causes large changes in the dynamics of both the phenylpropanoid and flavonoid pathways (Fig. 2B).

Epistatic effects

In both populations, analysis of potential epistatic interactions between all locus pairs (excluding those closely linked) revealed significant epistatic interactions for the synthesis of all traits except for chlorogenic acid.

Maysin

Two significant epistatic interactions were detected for the synthesis of maysin: *bnlg439* (*p*) × *umc1636* (*rem1*) in population 1 and *umc1963* (*mxm1*) × *umc1125* (bin 7.04) in population 2. The first epistatic interaction (*p* × *rem1*) was previously described by Byrne et al. (1996, 1998) as involving the main QTL for maysin synthesis; however, in our study, only minor QTLs mapped close to these loci. Unlike previous studies, our data do not show the dominant gene action for low maysin accumulation. Instead, the *rem1* allele from NC7A increases maysin levels in an additive manner in association with homozygous alleles at the *p* region from Tx501 (Table 5). Similar gene action at the *rem1* region has been reported by Szalma et al. (2002).

In the second interaction, *mxm1* × *umc1125* (bin 7.04) for population 2, *umc1125* only had a significant effect within the Mp708 *mxm1*-homozygous individuals. We are uncertain how these results should be interpreted in terms of molecular-level interactions or pathway dynamics.

Apimaysin

In both populations, the *pr1* locus was significant in the interactions between *umc1800* (*pr1*) and *umc1963* (*mxm1*) and between *mmc0282* (*pr1*) and *phi126* (*qmaysin9*) in populations 1 and 2, respectively. Genotype class mean values in both interactions indicate that apimaysin is synthesized only

Table 5. Genotype class means for apimaysin and methoxymaysin with significant epistatic interactions for the (Tx501 × NC7A)F₂ population.

Genotypes		Maysin (<i>p</i> × <i>rem1</i>)	Apimaysin (<i>pr1</i> × <i>mxm1</i>)	Methoxymaysin (<i>mxm1</i> × <i>umc1636</i>)
Locus 1 (L1)	Locus 2 (L2)	(L1 × L2)	(L1 × L2)	(L1 × L2)
Tx501	Tx501	0.227 <i>d</i>	0.000 <i>d</i>	0.055 <i>c</i>
	Heterozygote	0.363 <i>b</i>	0.006 <i>c</i>	0.079 <i>b</i>
	NC7A	0.552 <i>a</i>	0.007 <i>c</i>	0.111 <i>a</i>
Heterozygote	Tx501	0.291 <i>c</i>	0.007 <i>b,c</i>	0.033 <i>c,d</i>
	Heterozygote	0.312 <i>b</i>	0.012 <i>b</i>	0.045 <i>c</i>
	NC7A	0.292 <i>b,c</i>	0.018 <i>b</i>	0.047 <i>c</i>
NC7A	Tx501	0.162 <i>e</i>	0.034 <i>b</i>	0.025 <i>e</i>
	Heterozygote	0.239 <i>d</i>	0.196 <i>a</i>	0.033 <i>c,d</i>
	NC7A	0.241 <i>d</i>	0.164 <i>a</i>	0.038 <i>c</i>

Note: Within a column, means followed by common letters are not significantly different at $\alpha = 0.05$.

Table 6. Genotype class means for maysin, apimaysin, and methoxymaysin with significant epistatic interactions for the (Tx501 × Mp708)F₂ population.

				Methoxymaysin	
Genotypes		Maysin (<i>mxm1</i> × <i>umc1125</i>)	Apimaysin (<i>pr1</i> × <i>qmaysin9</i>)	(<i>mxm1</i> × <i>rem1</i>)	(<i>mxm1</i> × <i>umc1125</i>)
Locus 1 (L1)	Locus 2 (L2)	(L1 × L2)	(L1 × L2)	(L1 × L2)	(L1 × L2)
Tx501	Tx501	0.071 <i>d</i>	0.007 <i>d</i>	0.113 <i>a</i>	0.086 <i>a</i>
	Heterozygote	0.070 <i>d</i>	0.009 <i>cd</i>	0.083 <i>b</i>	0.090 <i>a</i>
	Mp708	0.087 <i>d</i>	0.008 <i>c</i>	0.070 <i>b</i>	0.100 <i>a</i>
Heterozygote	Tx501	0.273 <i>b</i>	0.010 <i>c</i>	0.032 <i>c</i>	0.026 <i>d</i>
	Heterozygote	0.275 <i>b</i>	0.008 <i>c</i>	0.030 <i>c</i>	0.030 <i>c</i>
	Mp708	0.232 <i>bc</i>	0.014 <i>c</i>	0.026 <i>d</i>	0.037 <i>b</i>
Mp708	Tx501	0.303 <i>b</i>	0.147 <i>a</i>	0.020 <i>de</i>	0.020 <i>e</i>
	Heterozygote	0.286 <i>b</i>	0.087 <i>b</i>	0.022 <i>d</i>	0.021 <i>e</i>
	Mp708	0.359 <i>a</i>	0.075 <i>b</i>	0.020 <i>de</i>	0.022 <i>e</i>

Note: Within a column, means followed by common letters are not significantly different at $\alpha = 0.05$.

when the *pr1* alleles from NC7A and Mp708 are homozygous and non-functional (Tables 5 and 6). Additionally, the *mxm1* allele from Tx501 had a negative effect in apimaysin synthesis in association with the non-functional *pr1* allele in population 1. This interaction is consistent with the general suppression by the Tx501 *mxm1* allele of all compounds except methoxymaysin.

In population 2, the highest levels of apimaysin were present in individuals homozygous for Mp708 alleles of *pr1* and homozygous for Tx501 alleles at *qmaysin9* (Table 6). Although only supported in the interaction and not as a main-effect QTL, the genotype means of the interaction are consistent with the expectation of a second hydroxylase gene. Thus, the position of *qmaysin9* is our best indication yet for second gene hydroxylase activity. To produce the maximum apimaysin level, one must be homozygous for Mp708 at *pr1* and homozygous for Tx501 at the location of the second hydroxylase activity, thereby eliminating the compensating locus from Mp708. This hypothesis will be tested in additional populations.

Methoxymaysin

Three significant epistatic interactions were detected for methoxymaysin synthesis: *umc1963* (*mxm1*) × *umc1636* (*rem1*) in population 1 and *umc1963* (*mxm1*) × *umc1370* (*rem1*) and *umc1963* (*mxm1*) × *umc1125* (bin 7.04) in population 2. Genotype class means for the *mxm1* × *rem1* interaction indicate

that methoxymaysin levels increase only when the *mxm1* allele from Tx501 is homozygous in association with the *rem1* allele from NC7A and Tx501 in populations 1 and 2, respectively (Tables 5 and 6). These results support two conclusions about *rem1*. First, by whatever mechanism *rem1* interacts with *p* to increase maysin, *rem1* may also interact with *mxm1* to increase methoxymaysin. This is in contrast to the lack of effect of *rem1* on apimaysin (Lee et al. 1998; Tables 1, 3, and 4 in this paper). Second, these results indicate that three alleles of *rem1* are present with the relative strength of NC7A > Tx501 > Mp708. Because the allele *rem1* has been previously reported to act in a dominant manner (Lee et al. 1988), *rem1* must be able to display different genetic actions depending on the background present. We must acknowledge that an alternative hypothesis is that there is more than one genetic factor on the short arm of chromosome 9 that affects maysin and methoxymaysin levels in these populations.

For the second epistatic interaction in population 2, *mxm1* × *umc1125* (bin7.04), the genotype class means indicate that the Mp708 allele at *umc1125* only increases methoxymaysin concentration when the *mxm1* allele from Tx501 is present.

Conclusions

Our data indicate that the synthesis of methoxymaysin in Tx501 occurs via alterations in both the flavone and phenyl-

propanoid pathways. These alternations are controlled by a major QTL locus we have designated *mxm1*. Because of the genetic effects of *mxm1* on both the synthesis of maysin and chlorogenic acid, *mxm1* may be a regulatory locus. This regulation is most likely to act at early steps of the phenylpropanoid and flavonoid pathways by altering the expression of specific isoforms of enzyme activities to redistribute the flow of specific intermediates through the pathway (Fig. 2B). However, *mxm1* may also be a structural gene that has the same effect of altering the flow of specific intermediates. If *mxm1* is a structural gene, it is most likely in the general phenylpropanoid pathway to explain *mxm1* effects on both chlorogenic acid and flavones. Although we do not know the positions in the pathway of the hydroxylase and methyltransferase activities, our data indicates that the synthesis of apimaysin, maysin, and methoxymaysin does not precede along a single linear pathway as depicted in Fig. 2A, but rather by multiple, interacting pathways (Fig. 2B). This is similar to the “matrix” representation of the phenylpropanoid and flavonoid pathways presented by Grotewold et al. (1998).

Our data also showed that a non-functional *pr1* allele is required for the synthesis of apimaysin, supporting the involvement of this locus in the hydroxylation of the B ring in flavone synthesis (Styles and Cezka 1975). However, the high levels of maysin in many individuals homozygous recessive for *pr1* indicate the presence of alternative hydroxylase enzymes in the flavone pathway.

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